IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Kutchan et al

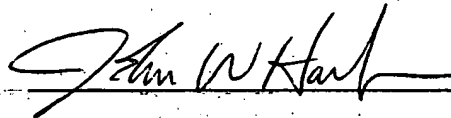Serial No.: 09/937,665   Art Unit:

Filed   :   September 26, 2001   Examiner:

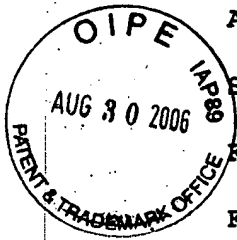For   :   CODEINONE REDUCTASE FROM ALKALOID POPPY

COPY

VERIFIED STATEMENT UNDER 37 CFR §1.821(f)

I hereby verify that the computer readable diskette enclosed herewith includes the same information as provided in the Sequence Listing of the subject application, and that this statement is made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of the application or any patent issued thereon.

John W. Harbour
Johnson & Johnson
One Johnson & Johnson Plaza
New Brunswick, NJ 08933
DATE: January 2, 2002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Kutchan et al

Serial No.: 09/937,665   Art Unit:

Filed   : September 26, 2001   Examiner:

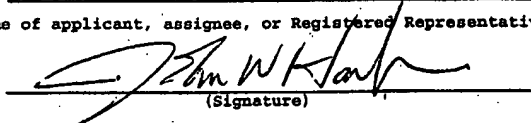For   : CODEINONE REDUCTASE FROM ALKALOID POPPY

I hereby certify that this correspondence is being deposited with the
United States Postal Service as first class mail in an envelope addressed
to: Assistant Commissioner for Patents, Washington, D.C. 20231 on

January 2, 2002
_____
(Date)

John W. Harbour
Name of applicant, assignee, or Registered Representative

_____
(Signature)

January 2, 2002
_____
(Date of Signature)

Assistant Commissioner for Patents
Washington, D.C. 20231

RESPONSE

Pursuant to the requirements of 37 CFR 1.822 and/or 1.823 and further to the Formalities Letter mailed 11/1/01, Applicant submits a corrected copy of the Sequence Listing in computer readable form, including a printed version of the Sequence Listing of the subject application. As required, a copy of the Formalities Letter is also attached.

Furthermore, a Verified Statement concerning the enclosed diskette is submitted herewith.

If any fees are due in connection with the filing of this response, authorization is hereby given to charge the amount

of such fee to Depot Account No. 10-0750/J&J825/JWH in the name of Johnson & Johnson.

Respectfully submitted,

John W. Harbour
Reg. No.31,365
Attorney for Applicants

Johnson & Johnson
One Johnson & Johnson Plaza
New Brunswick, NJ 08933-7003
(732)524-2169
DATE: January 2, 2002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Kutchan et al

Serial No.: 09/937,665   Art Unit:

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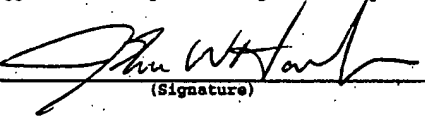For   : CODEINONE REDUCTASE FROM ALKALOID POPPY

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United States Postal Service as first class mail in an envelope addressed
to: Commissioner of Patents, Washington, D.C. 20231 on

January 2, 2002
(Date of Deposit)

John W. Harbour
(Name of applicant, assignee, or Registered Representative)

(Signature)

January 2, 2002
(Date of Signature)

Honorable Commissioner of Patents
Washington, D.C. 20231

AMENDMENT

Dear Sir:

In response to the Formalities Letter of November 1, 2001, please amend the above-identified application as follows:

In the Specification:

Please replace the paragraph beginning at page 8, line 31, with the following rewritten paragraph:

--Figure 2.   Partial amino acid sequences of native codeinone reductase. Peptide 3 is SEQ ID NO: 9, Peptide 7 is SEQ ID NO: 10, Peptide 14 is SEQ ID NO: 11, Peptide 16 is SEQ ID NO: 12,

Peptide 17 is SEQ ID NO: 13, Peptide 25 is SEQ ID NO: 14, and
Peptide 29 is SEQ ID NO: 15.--

Please replace the paragraph beginning at page 9, line 7, with the
following rewritten paragraph:

--   Codeinone reductase peptides 3, 7, 14, 16, and 17 aligned
with the reductase subunit of the 6'-deoxychalcone synthase
complex from alfalfa (SEQ ID NO: 16), glycyrrhiza (SEQ ID NO: 17)
and soybean (SEQ ID NO: 18) allowing the relative positioning of
these internal peptides from opium poppy (SEQ ID NO: 19).--

Please replace the paragraphs beginning at page 10, line 26, with
the following rewritten paragraphs:

--   **Figure 10.** cDNA sequence of *corl.1.* (SEQ ID NO: 20)
       Figure 11. cDNA sequence of *corl.2.* (SEQ ID NO: 21)
       Figure 12. cDNA sequence of *corl.3.* (SEQ ID NO: 22)
       Figure 13. cDNA sequence of *corl.4.* (SEQ ID NO: 23)
       Figure 14. Partial cDNA sequence of *corl.5.* (SEQ ID NO: 24)
       Figure 15. Partial cDNA sequence of *corl.6.* (SEQ ID NO: 25)

--

Please replace the text beginning at page 12, line 1 and ending
with line 22, with the following rewritten text:

--

SEQ ID NO: 1

5'-GAA CTT TTT ATA ACT TCT AA-3' (derived from Peptide 14) and
      G   C   C   C   G   C
                  T

SEQ ID NO: 2

```
3'-GTG GTC TAA CGT CAI CGT TCI CCT TT-5' (derived from Peptide 7)
     A                 A G     C
```

Resolution of an aliquot of the first PCR experiment by agarose gel electrophoresis revealed a mixture of DNA products, none of which was the expected band of approximately 480 bp. This was presumably due to the relatively low specificity of the degenerate primers coupled to a low abundance of codeinone reductase transcript. Another aliquot of the first PCR reaction mixture was, therefore, used as template for nested PCR with the following primers:

SEQ ID NO: 1

```
5'-GAA CTT TTT ATA ACT TCT AA-3' (same as Peptide 14 primer above) and
    G   C   C   C  G  C
                T
```

SEQ ID NO: 3

```
3'-CAI CAC TTA GTT CAC CTT TAC-5' (nested primer derived from Peptide 16)
       G       C       C
```

to yield an approximately 360 bp DNA fragment and the following primers to yield an approximately 180 bp DNA product:

SEQ ID NO: 4

```
5-'GTI GTI AAC CAA GTI GAA ATG AGI CCI AC-3' (nested primer derived from
        T   G       G    TC              Peptide 16) and
```

SEQ ID NO: 2

```
3'-GTG GTC TAA CGT CAI CGT TCI CCT TT-5' (same as Peptide 7 primer above)
    A                 A G     C
```

--

- 3 -

Please replace the text beginning at page 13, line 8 and ending at line 14, with the following rewritten text:

--

SEQ ID NO: 5

5'-ATG GAG AGT AAT GGT GTA CCT-3' (located at the 5'-terminus) and

SEQ ID NO: 6

3'-TCT ACC ATT CAC TCC TGA CAG-5' (located in the 3'-flanking region)

followed by nested PCR with the following primer pair:

SEQ ID NO: 7

5'-ATG GCT AGC ATG GAG AGT AAT GGT GTA CCT ATG-3' (located at the
      Nhe 1   5'-terminus) and

SEQ ID NO: 8

3'-CTT CTC AAG ACC CTA CTC TTC CTA CCT AGG GAA-5' (located at the
                                   Bam HI   3'-terminus).--

REMARKS/ARGUMENTS

Amendments have been made to the specification and sequences from the text and figures have been incorporated into the sequence listing. In response to the Formalities Letter of November 1, 2001, applicants include with this response a Sequence listing and a Computer Readable Form of the Sequence Listing. The undersigned hereby states that the Paper Copy and the Computer Readable Form submitted in accordance with 37 CFR§ 1.821 are identical. No new matter has been added by these amendments.

Attached hereto is a marked-up version of the changes made to the specification by the current amendment. The attached page(s) is/are captioned "Version with markings to show changes made". Favorable consideration is respectfully requested.

Respectfully submitted,

By: _____

John W. Harbour
Reg. No. 31,365

Johnson & Johnson
One Johnson & Johnson Plaza
New Brunswick, NJ 08933-7003
(732) 524-2169
Dated: January 2, 2002

In the Specification:

The paragraph beginning at page 8, line 31, has been replaced with
the following rewritten paragraph:

--Figure 2.   Partial amino acid sequences of native codeinone
reductase.   Peptide 3 is SEO ID NO: 9, Peptide 7 is SEO ID NO:
10, Peptide 14 is SEO ID NO: 11, Peptide 16 is SEO ID NO: 12,
Peptide 17 is SEO ID NO: 13, Peptide 25 is SEO ID NO: 14, and
Peptide 29 is SEO ID NO: 15.--

The paragraph beginning at page 9, line 7, has been replaced with
the following rewritten paragraph:

-- Codeinone reductase peptides 3, 7, 14, 16, and 17 aligned
with the reductase subunit of the 6'-deoxychalcone synthase
complex from alfalfa (SEO ID NO: 16), glycyrrhiza (SEO ID NO: 17)
and soybean (SEO ID NO: 18) allowing the relative positioning of
these internal peptides from opium poppy (SEO ID NO: 19).--

The paragraphs beginning at page 10, line 26, have been replaced
with the following rewritten paragraphs:

--   Figure 10.cDNA sequence of corl.1. (SEO ID NO: 20)
       Figure 11.cDNA sequence of corl.2. (SEO ID NO: 21)
       Figure 12.cDNA sequence of corl.3. (SEO ID NO: 22)
       Figure 13.cDNA sequence of corl.4. (SEO ID NO: 23)
       Figure 14.Partial cDNA sequence of corl.5. (SEO ID NO: 24)
       Figure 15.Partial cDNA sequence of corl.6. (SEO ID NO: 25)

   --

The text beginning at page 12, line 1 and ending with line 22, has been replaced with the following rewritten text:

--

<u>SEQ ID NO: 1</u>

5'-GAA CTT TTT ATA ACT TCT AA-3' (derived from Peptide 14) and
    G   C   C   C   G .C
              T

<u>SEQ ID NO: 2</u>

3'-GTG GTC TAA CGT CAI CGT TCI CCT TT-5' (derived from Peptide 7)
    A   A G   C

Resolution of an aliquot of the first PCR experiment by

agarose gel electrophoresis revealed a mixture of DNA products,

none of which was the expected band of approximately 480 bp.

This was presumably due to the relatively low specificity of the

degenerate primers coupled to a low abundance of codeinone

reductase transcript. Another aliquot of the first PCR reaction

mixture was, therefore, used as template for nested PCR with the

following primers:

<u>SEQ ID NO: 1</u>

5'-GAA CTT TTT ATA ACT TCT AA-3' (same as Peptide 14 primer above) and
    G   C .C   C   G   C
            T

<u>SEQ ID NO: 3</u>

3'-CAI CAC TTA GTT CAC CTT TAC-5' (nested primer derived from Peptide 16)
      G   C   C

to yield an approximately 360 bp DNA fragment and the following

primers to yield an approximately 180 bp DNA product:

- 7 -

<u>SEQ ID NO: 4</u>

5-'GTI GTI AAC CAA GTI GAA ATG AGI CCI AC-3'   (nested primer derived from
    T G   G TC   Peptide 16) and

<u>SEQ ID NO: 2</u>

3'-GTG GTC TAA CGT CAI CGT TCI CCT TT-5'   (same as Peptide 7 primer above)
  A   A G C

--

The text beginning at page 13, line 8 and ending at line 14, has
been replaced with the following rewritten text:

--

<u>SEQ ID NO: 5</u>

5'-ATG GAG AGT AAT GGT GTA CCT-3'   (located at the 5'-terminus) and

<u>SEQ ID NO: 6</u>

3'-TCT ACC ATT CAC TCC TGA CAG-5'   (located in the 3'-flanking region)

followed by nested PCR with the following primer pair:

<u>SEQ ID NO: 7</u>

5'-ATG GCT AGC ATG GAG AGT AAT GGT GTA CCT ATG-3'   (located at the
    Nhe 1   5'-terminus) and

<u>SEQ ID NO: 8</u>

3'-CTT CTC AAG ACC CTA CTC TTC CTA CCT AGG GAA-5'   (located at the
                 Bam HI   3'-terminus).--

SEQUENCE LISTING

<110> Toni, Kutchan
       Anthony, Fist
       David, Atkins
       Meinhart, Zenk

<120> CODEINONE REDUCTASE FROM ALKALOID POPPY

<130> J&J-1825

<140> 09/937665
<141> 2001-09-26

<150> PCT/AU00/00249
<151> 2000-03-24

<150> AU PP 9463
<151> 1999-03-26

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<170> PatentIn version 3.1

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4

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<220>
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1               5                   10



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1               5


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Xaa Val Ala Gln Val
1           5


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<211> 230
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Gln Ala Leu Gly Glu Ala Leu Lys Glu Ala Ile Glu Leu Gly Leu Val
            20                  25                  30


Thr Arg Glu Glu Leu Phe Val Thr Ser Lys Leu Trp Val Thr Glu Asn
            35                  40                  45


His Pro His Leu Val Ile Pro Ala Leu Gln Lys Ser Leu Lys Thr Leu
    50                  55                  60


Gln Leu Asp Tyr Leu Asp Leu Tyr Leu Ile His Trp Pro Leu Ser Ser
65                  70                  75                  80


Gln Pro Gly Lys Phe Ser Phe Pro Ile Asp Val Ala Asp Leu Leu Pro
                85                  90                  95


Phe Asp Val Lys Gly Val Trp Glu Ser Met Glu Glu Ser Leu Lys Leu
            100                 105                 110


Gly Leu Thr Lys Ala Ile Gly Val Ser Asn Phe Ser Val Lys Lys Leu
            115                 120                 125


Glu Asn Leu Leu Ser Val Ala Thr Val Leu Pro Ala Val Asn Gln Val
        130                 135                 140


Glu Met Asn Leu Ala Trp Gln Gln Lys Lys Leu Arg Glu Phe Cys Asn
145                 150                 155                 160


Ala Asn Gly Ile Val Leu Thr Ala Phe Ser Pro Leu Arg Lys Gly Ala
                165                 170                 175


Ser Arg Gly Pro Asn Glu Val Met Glu Asn Asp Met Leu Lys Glu Ile
            180                 185                 190
```

```
Ala Asp Ala His Gly Lys Ser Val Ala Gln Ile Ser Leu Arg Trp Leu
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Tyr Glu Gln Gly Val Thr Phe Val Pro Lys Ser Tyr Asp Lys Glu Arg
        210                 215                 220


Met Asn Gln Asn Leu Cys
225                 230


<210>   17
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        lycyrrhiza

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1               5                   10                  15


Thr Ala Leu Gly Glu Ala Leu Lys Glu Ala Arg Asp Leu Gly Leu Val
            20                  25                  30


Thr Arg Glu Glu Leu Phe Val Thr Ser Lys Leu Trp Val Thr Glu Asn
        35                  40                  45


His Pro His Leu Val Ile Pro Ala Leu Arg Lys Ser Leu Glu Thr Leu
        50                  55                  60


Gln Leu Glu Tyr Leu Asp Leu Tyr Leu Ile His Trp Pro Leu Ser Ser
65                  70                  75                  80


Gln Pro Gly Lys Phe Ser Phe Pro Ile Gln Val Glu Asp Leu Leu Pro
                85                  90                  95


Phe Asp Val Lys Gly Val Trp Glu Ser Met Glu Glu Cys Leu Lys Leu
            100                 105                 110


Gly Leu Thr Lys Ala Ile Gly Val Ser Asn Phe Ser Val Lys Lys Leu
        115                 120                 125


Gln Asn Leu Leu Ser Val Ala Thr Ile Arg Pro Ala Val Asn Gln Val
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Glu Met Asn Leu Ala Trp Gln Gln Lys Lys Leu Arg Glu Phe Cys Thr
145   150   155   160

Ala Asn Gly Ile Val Leu Thr Ala Phe Ser Pro Leu Arg Lys Gly Ala
                165   170   175

Ser Arg Gly Pro Asn Glu Val Met Glu Asn Asp Met Leu Lys Gly Ile
            180   185   190

Ala Glu Ala His Gly Lys Ser Ile Ala Gln Val Ser Leu Arg Trp Leu
            195   200   205

Tyr Glu Gln Gly Val Thr Phe Val Ala Lys Ser Tyr Asp Lys Glu Arg
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Met Asn Gln Asn Leu Gln
225   230

<210> 18
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Gln Ala Leu Gly Glu Ala Leu Lys Glu Ala Ile His Leu Gly Leu Val
            20   25   30

Ser Arg Gln Asp Leu Phe Val Thr Ser Lys Leu Trp Val Thr Glu Asn
            35   40   45

His Pro His Leu Val Leu Pro Ala Leu Arg Lys Ser Leu Lys Thr Leu
        50   55   60

Gln Leu Glu Tyr Leu Asp Leu Tyr Leu Ile His Trp Pro Leu Ser Ser
65   70   75   80

```
Gln Pro Gly Lys Phe Ser Phe Pro Ile Glu Val Glu Asp Leu Leu Pro
              85                  90                  95

Phe Asp Val Lys Gly Val Trp Glu Ser Met Glu Glu Cys Gln Lys Leu
             100                 105                 110

Gly Leu Thr Lys Ala Ile Gly Val Ser Asn Phe Ser Val Lys Lys Leu
         115                 120                 125

Gln Asn Leu Leu Ser Val Ala Thr Ile Arg Pro Val Val Asp Gln Val
     130                 135                 140

Glu Met Asn Leu Ala Trp Gln Gln Lys Lys Leu Arg Glu Phe Cys Lys
145                 150                 155                 160

Glu Asn Gly Ile Ile Val Thr Ala Phe Ser Pro Leu Arg Lys Gly Ala
             165                 170                 175

Ser Arg Gly Pro Asn Glu Val Met Glu Asn Asp Val Leu Lys Glu Ile
         180                 185                 190

Ala Glu Ala His Gly Lys Ser Ile Ala Gln Val Ser Leu Arg Trp Leu
     195                 200                 205

Tyr Glu Gln Gly Val Thr Phe Val Pro Lys Ser Tyr Asp Lys Glu Arg
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Met Asn Gln Asn Leu His
225                 230
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      pium poppy

<400> 19

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1                 5                  10                  15

Val Val Asn Gln Val Glu Met Ser Pro Thr Leu Val Leu His Gln Ile
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11

Ala Val Ala Arg Gly Lys Val Asn Glu Ile Pro Lys
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            20   25   30

Ala Cys Thr Cys Thr Cys Ala Gly Thr Thr Cys Cys Gly Gly Cys Ala
        35   40   45

Thr Thr Cys Gly Gly Ala Thr Gly Cys Cys Thr Gly Cys Thr Thr Thr
    50   55   60

Ala Gly Gly Thr Ala Thr Gly Gly Gly Ala Ala Cys Ala Gly Cys Thr
65   70   75   80

Gly Ala Ala Ala Cys Ala Ala Thr Gly Gly Thr Ala Ala Ala Ala Gly
            85   90   95

Gly Ala Ala Cys Ala Gly Ala Ala Ala Gly Ala Gly Ala Gly Ala Ala
        100   105   110

Ala Thr Thr Gly Gly Cys Gly Thr Thr Thr Thr Thr Gly Ala Ala Ala
        115   120   125

12

Gly Cys Gly Ala Thr Ala Gly Ala Gly Gly Thr Cys Gly Gly Thr Thr
   130   135   140

Ala Cys Ala Gly Ala Cys Ala Cys Thr Thr Cys Gly Ala Thr Ala Cys
145   150   155   160

Ala Gly Cys Thr Gly Cys Thr Gly Cys Ala Thr Ala Cys Cys Ala Ala
         165   170   175

Ala Cys Thr Gly Ala Ala Gly Ala Gly Thr Gly Thr Cys Thr Thr Gly
     180   185   190

Gly Thr Gly Ala Ala Gly Cys Thr Ala Thr Ala Gly Cys Thr Gly Ala
    195   200   205

Ala Gly Cys Ala Cys Thr Thr Cys Ala Ala Cys Thr Thr Gly Gly Thr
   210   215   220

Cys Thr Ala Ala Thr Ala Ala Ala Ala Thr Cys Thr Cys Gly Ala Gly
225   230   235   240

Ala Thr Gly Ala Ala Cys Thr Cys Thr Thr Cys Ala Thr Cys Ala Cys
        245   250   255

Thr Thr Cys Cys Ala Ala Gly Cys Thr Cys Thr Gly Gly Thr Gly Cys
      260   265   270

Gly Cys Thr Gly Ala Thr Gly Cys Thr Cys Ala Cys Gly Cys Thr Gly
     275   280   285

Ala Thr Cys Thr Thr Gly Thr Cys Cys Thr Cys Cys Cys Thr Gly Cys
   290   295   300

Thr Cys Thr Thr Cys Ala Gly Ala Ala Thr Thr Cys Thr Cys Thr Gly
305   310   315   320

Ala Gly Gly Ala Ala Thr Cys Thr Thr Ala Ala Ala Thr Thr Gly Gly
        325   330   335

Ala Cys Thr Ala Thr Cys Thr Thr Gly Ala Thr Cys Thr Ala Thr Ala
      340   345   350

13

```
Thr Thr Thr Gly Ala Thr Ala Cys Ala Cys Cys Ala Thr Cys Cys Gly
        355                 360                 365

Gly Thr Ala Ala Gly Cys Thr Thr Gly Ala Ala Gly Cys Cys Ala Gly
        370                 375                 380

Gly Gly Ala Ala Gly Thr Thr Thr Gly Thr Thr Ala Ala Cys Gly Ala
385                 390                 395                 400

Ala Ala Thr Ala Cys Cys Ala Ala Ala Gly Gly Ala Thr Cys Ala Thr
                405             410                 415

Ala Thr Cys Cys Thr Thr Cys Cys Ala Ala Thr Gly Gly Ala Cys Thr
            420                 425                 430

Ala Cys Ala Ala Ala Thr Cys Thr Gly Thr Ala Thr Gly Gly Gly Cys
        435                 440                 445

Ala Gly Cys Cys Ala Thr Gly Gly Ala Ala Gly Ala Gly Thr Gly Thr
    450                 455                 460

Cys Ala Gly Ala Cys Cys Cys Thr Thr Gly Gly Cys Thr Thr Cys Ala
465                 470                 475                 480

Cys Thr Ala Gly Gly Gly Cys Ala Ala Thr Cys Gly Gly Gly Gly Thr
                485             490                 495

Cys Thr Gly Thr Ala Ala Thr Thr Thr Cys Thr Cys Ala Thr Gly Cys
        500                 505                 510

Ala Ala Ala Ala Gly Gly Cys Thr Thr Cys Ala Ala Gly Ala Gly Thr
        515                 520                 525

Thr Gly Ala Thr Gly Gly Ala Ala Ala Cys Ala Gly Cys Cys Ala Ala
        530                 535                 540

Cys Ala Gly Cys Cys Cys Thr Cys Cys Ala Gly Thr Thr Gly Thr Gly
545                 550                 555                 560

Ala Ala Thr Cys Ala Ala Gly Thr Gly Gly Ala Gly Ala Thr Gly Ala
                565             570                 575

Gly Cys Cys Cys Gly Ala Cys Thr Thr Thr Ala Cys Ala Thr Cys Ala
```

580   585   590

Ala Ala Ala Ala Ala Ala Thr Cys Thr Gly Ala Gly Gly Gly Ala Ala
         595   600   605

Thr Ala Thr Thr Gly Cys Ala Ala Gly Gly Cys Cys Ala Ala Thr Ala
    610   615   620

Ala Thr Ala Thr Cys Ala Thr Gly Ala Thr Cys Ala Cys Cys Gly Cys
625   630   635   640

Ala Cys Ala Cys Thr Cys Ala Gly Thr Thr Thr Gly Gly Gly Ala
             645   650   655

Gly Cys Cys Gly Thr Ala Gly Gly Thr Gly Cys Cys Gly Cys Cys Thr
             660   665   670

Gly Gly Gly Gly Cys Ala Cys Cys Ala Ala Thr Gly Cys Ala Gly Thr
         675   680   685

Thr Ala Thr Gly Cys Ala Thr Thr Cys Thr Ala Ala Gly Gly Thr Gly
    690   695   700

Cys Thr Thr Cys Ala Cys Cys Ala Gly Ala Thr Thr Gly Cys Thr Gly
705   710   715   720

Thr Gly Gly Cys Cys Ala Gly Ala Gly Gly Ala Ala Ala Ala Thr Cys
         725   730   735

Thr Gly Thr Thr Gly Cys Cys Cys Ala Gly Gly Thr Thr Ala Gly Thr
         740   745   750

Ala Thr Gly Ala Gly Ala Thr Gly Gly Gly Thr Thr Thr Ala Cys Cys
    755   760   765

Ala Gly Cys Ala Ala Gly Gly Cys Gly Cys Gly Ala Gly Thr Cys Thr
    770   775   780

Thr Gly Thr Gly Gly Thr Gly Ala Ala Ala Ala Gly Thr Thr Thr Cys
785   790   795   800

Ala Ala Thr Gly Ala Ala Gly Cys Gly Ala Gly Gly Ala Thr Gly Ala
         805   810   815

                                                                    15

```
Ala Gly Gly Ala Ala Ala Ala Cys Cys Thr Thr Ala Ala Gly Ala Thr
        820                 825             830

Ala Thr Thr Thr Gly Ala Thr Thr Gly Gly Gly Ala Ala Cys Thr Ala
        835                 840             845

Ala Cys Gly Gly Cys Ala Gly Ala Ala Gly Ala Cys Ala Thr Gly Gly
    850                 855             860

Ala Ala Ala Ala Gly Ala Thr Cys Ala Gly Thr Gly Ala Gly Ala Thr
865             870             875                 880

Thr Cys Cys Ala Cys Ala Ala Thr Cys Thr Ala Gly Ala Ala Cys Ala
            885                 890             895

Ala Gly Cys Thr Cys Thr Gly Cys Thr Gly Cys Thr Thr Thr Cys Thr
        900             905             910

Thr Gly Thr Thr Ala Thr Cys Ala Cys Cys Gly Ala Cys Thr Gly Gly
    915                 920             925

Ala Cys Cys Thr Thr Thr Cys Ala Ala Ala Ala Cys Thr Gly Ala Ala
    930             935             940

Gly Ala Ala Gly Ala Gly Thr Thr Cys Thr Gly Gly Gly Ala Thr Gly
945             950             955                 960

Ala Gly Ala Ala Gly Gly Ala Thr Thr Gly Ala Ala Ala Cys Ala Thr
            965             970             975

Cys Ala Ala Thr Thr Ala Thr Ala Gly Ala Thr Gly Gly Thr Ala Ala
        980             985             990

Gly Thr Gly Ala Gly Gly Ala Cys  Thr Gly Thr Cys Ala  Ala Ala Ala
        995             1000            1005

Ala Ala  Gly Thr Ala Ala Thr  Cys Ala Gly Thr Thr  Thr Thr Thr
    1010            1015            1020

Cys Cys  Cys Thr Cys Cys Gly  Thr Thr Thr Thr Gly
    1025            1030            1035
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<212>  DNA
<213>  papaver somniferum

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<223>  codeinone reductase isoform


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ggtatgggaa cagttgaaac aatggaaaag gaacagaaa gagagaaatt ggcgttttg      120

aatgcgatag aggtcggtta cagacacttc gatacagctg ctgcatacca aagtgaagag     180

tgtcttggtg aagctatagc tgaagcactt caacttggtt taataaaatc tcgagatgaa     240

ctcttcatca cttccaagct ctggtgcgct gatgctcacg ctgatcttgt cctccctgct     300

cttcagaatt ctctgaggaa tctcaaattg gagtaccttg atctatattt gatacaccat     360

ccggtaagct tgaagccagg gaagcttgtt aacgaaatac caaaggatca tattcttcca     420

atggactaca atctgtatg ggcagccatg gaagagtgtc agacccttgg cttcactagg     480

gcaatcggtg tcagtaattt ctcatgcaaa aagcttcaag agttgatggc aacagccaag     540

atccctccag ttgtgaatca agtggagatg agcccgactt tacatcaaaa aaatctgagg     600

gaatattgca aggccaataa tatcatgatc actgcacact cggttttggg agccataggt     660

gctccatggg gcagcaacgc agttatggat tctaaggtgc ttcaccagat tgctgtggca     720

agaggaaaat ctgttgccca ggttagtatg agatgggttt accagcaagg cgcgagtctt     780

gtggtgaaaa gtttcaatga agcgaggatg aaggaaaacc ttaagatatt tgattcggaa     840

ctaacggcag aagatatgga aaagatcagt gagattccgc aatctagaac aagctctgct     900

gatttcttgt tatcaccgac tggacctttc aaaactgaag aagagttctg ggatgagaag     960

gattga                                                                 966


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17

<400> 22

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aaagcgatag aggtcggtta cagacacttc gatacagctg ctgcatacca aagtgaagag     180

tgtcttggtg aagctatagc tgaagcactt caacttggtc taataaaatc tcgagatgaa     240

ctcttcatca cttccaagct ctggtgcgct gatgctcacg ctgatcttgt cctccctgct     300

cttcagaatt ctctgaggaa tcttaaattg gactatcttg atctatattt gatacaccat     360

ccggtaagct tgaagccagg gaagtttgtt aacgaaatac caaaggatca tatccttcca     420

atggactaca aatctgtatg ggcagccatg gaagagtgtc agacccttgg cttcactagg     480

gcaatcgggg tctgtaattt ctcatgcaaa aagcttcaag agttgatggc agcagccaag     540

atccctccag ttgtgaatca agtggagatg agcccgactt acatcaaaaa aaatctgagg     600

gaatattgca aggccaataa tatcatgatc actgcacact cggttttggg agccatatgt     660

gctccatggg gcagcaatgc agttatggat tctaaggtgc ttcaccagat tgctgtggca     720

agaggaaaat ctgttgccca ggttagtatg agatgggttt accagcaagg cgcgagtcta     780

gtggtgaaaa gtttcaatga agggaggatg aaggaaaacc ttaagatatt tgattgggaa     840

ctaacggcag agaatatgga aaagatcagt gagattccgc aatctagaac aagctctgct     900

gatttcttgt tatcaccgac tggacctttc aaaactgaag aagagttctg ggatgagaag     960

gattga                                                                966
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<223> codeinone reductase isoform

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aaagcgatag aggtcggtta cagacacttc gatacagctg ctgcatacca aagtgaagag     180

tgtcttggtg aagctatagc tgaagcactt caacttggtt taataaaatc tcgagatgaa     240
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18

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ctcttcatca cttccaagct ctggtgcgct gatgctcacg ctgatcttgt cctccctgct        300

cttcagaatt ctctgaggaa tctcaaattg gagtatcttg atctatattt gatacaccat        360

ccggtaagct tgaagccagg gaaatttgtt aacgaaatac caaaggatca tattcttcca        420

atggactaca aatctgtatg ggcagccatg gaagagtgtc agacccttgg cttcactagg        480

gcaatcggtg tcagtaattt ctcatgcaaa aagcttcaag agttgatggc agcagccaag        540

atccctccag ttgtgaatca agtggagatg agccctactt tacatcaaaa aaatctgagg        600

gaatattgca aggccaataa tatcatgatc actgcacact cggttttggg agccataggt        660

gctccatggg gcagcaatgc agttatggat tctaaggtgc ttcaccagat tgctgtggca        720

agaggaaaat ctgttgccca ggttagtatg agatgggttt accagcaagg cgcgagtctt        780

gtggtgaaaa gtttcaatga agggaggatg aaggaaaacc ttaagatatt tgattgggaa        840

ctaacggcag aagatatgga aaagatcagt gagattccgc aatctagaac aagctctgct        900

gctttcttgt tatcaccgac tggacctttc aaaactgaag aagagttctg ggatgagaag        960

gattga                                                                   966


<210> 24
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<212> DNA
<213> papaver somniferum

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<223> partial sequence of codeinone reductase isoform


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caaggccaat aatatcatga tcactgcaca ctcggttttg ggagccatag gtgctccatg        120

gggcagcaat gcagttatgg attctaaggt gctt                                    154


<210> 25
<211> 291
<212> DNA
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<220>
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<222> (1)..(291)
<223> partial sequence codeinone reductase isoform
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gggaagcttg ttaacgaaat accaaaggat catattcttc caatggacta caaatctgta     180

tgggcagcca tggaagagtg tcagaccctt ggcttcacta gggcaatcgg tgtcagtaat     240

ttctcatgca aaaagcttca agagttgatg gcaacagcca agatccctcc a             291
```

20

2

Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

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Molecular
Cloning
A LABORATORY MANUAL
SECOND EDITION

HYBRIDIZATION OF RADIOLABELED PROBES
TO IMMOBILIZED NUCLEIC ACIDS

There are many methods available to hybridize radioactive probes in solution to nucleic acids immobilized on solid supports such as nitrocellulose filters or nylon membranes. These methods differ in the following respects:

- Solvent and temperature used (e.g., 68°C in aqueous solution or 42°C in 50% formamide)

- Volume of solvent and length of hybridization (large volumes for periods as long as 3 days or minimal volumes for periods as short as 4 hours)

- Degree and method of agitation (continuous shaking or stationary)

- Use of agents such as Denhardt's reagent or BLOTTO to block the non-specific attachment of the probe to the surface of the solid matrix

- Concentration of the labeled probe and its specific activity

- Use of compounds, such as dextran sulfate (Wahl et al. 1979) or poly-ethylene glycol (Renz and Kurz 1984; Amasino 1986), that increase the rate of reassociation of nucleic acids

- Stringency of washing following the hybridization

Although the choice depends to a large extent on personal preference, we offer the following guidelines for choosing among the various methods available.

1. Hybridization reactions in 50% formamide at 42°C are less harsh on nitrocellulose filters than is hybridization at 68°C in aqueous solution. However, it has been found that the kinetics of hybridization in 80% formamide are approximately four times slower than in aqueous solution (Casey and Davidson 1977). Assuming a linear relationship between the rate of hybridization and the formamide concentration, the rate in 50% formamide should be two to three times slower than the rate in aqueous solution. Both types of solvents give excellent results and neither has a clear-cut advantage over the other.

2. The smaller the volume of hybridization solution, the better. In small volumes of solution, the kinetics of nucleic acid reassociation are faster and the amount of probe needed can be reduced so that the DNA on the filter acts as the driver for the reaction. However, it is essential that sufficient liquid be present for the filters to remain covered at all times by a film of the hybridization solution.

3. Continual movement of the probe solution across the filter is unnecessary, even for a reaction driven by the DNA immobilized on the filter. However, if a large number of filters are hybridized simultaneously, agitation is advisable to prevent the filters from adhering to one another.

4. The kinetics of the hybridization reaction are difficult to predict from theoretical considerations, partly because the exact concentration of the

immobilized nucleic acid and its availability for hybridization are unknown. When using probes that have the capacity to self-anneal (e.g., nick-translated double-stranded DNA), the following rule of thumb is useful: Allow the hybridization to proceed for a time sufficient to enable the probe in solution to achieve $1-3 \times C_o t_{1/2}$. In 10 ml of hybridization solution, 1 μg of a probe of 5-kb complexity will reach $C_o t_{1/2}$ in 2 hours. To determine the time of half-renaturation for any other probe, simply enter the appropriate values into the following equation:

$$1/x \times y/5 \times z/10 \times 2 = \text{number of hours to achieve } C_o t_{1/2}$$

where x = the weight of the probe added (in micrograms), y = its complexity (for most probes, complexity is proportional to the length of the probe in kilobases), and z = the volume of the reaction (in milliliters).

After hybridization to $3 \times C_o t_{1/2}$ has been reached, the amount of probe available for additional hybridization to the filter is negligible. For probes that do not have the capacity to self-anneal (e.g., RNA probes synthesized in vitro by bacteriophage-encoded DNA-dependent RNA polymerases; see Chapter 10), the hybridization time may be shortened, since the lack of a competing reaction in the solution favors hybridization of the probe to the DNA immobilized on the filter.

5. Several different types of agents can be used to block the nonspecific attachment of the probe to the surface of the filter. These include Denhardt's reagent (Denhardt 1966), heparin (Singh and Jones 1984), and nonfat dried milk (Johnson et al. 1984). Frequently, these agents are used in combination with denatured, fragmented salmon sperm or yeast DNA and detergents such as SDS. In our experience, virtually complete suppression of background hybridization is obtained by prehybridizing filters with a blocking agent consisting of 5× Denhardt's reagent, 0.5% SDS, and 100 μg/ml denatured, fragmented DNA. We recommend this mixture whenever the signal-to-noise ratio is expected to be low, for example, when carrying out northern analysis of low-abundance mRNAs or Southern hybridizations with single-copy sequences of mammalian DNA. However, in most other circumstances (Grunstein/Hogness hybridization [1975], Benton/Davis hybridization [1977], Southern hybridization [1975] of abundant DNA sequences, etc.), we recommend using 0.25% nonfat dried milk (0.05× BLOTTO; Johnson et al. 1984). This is much less expensive, easier to use than Denhardt's reagent, and, for these purposes, gives results that are equally satisfactory. In general, Denhardt's reagent is more effective for nylon membranes. The signal-to-noise ratio obtained with most brands of nylon membranes is higher with Denhardt's reagent than with BLOTTO. Nonfat dried milk is not recommended when using RNA probes or when carrying out northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase activity. For more information about blocking agents, see Table 9.1.

6. Blocking agents are usually included in both the prehybridization and hybridization solutions when nitrocellulose filters are used. However, when the nucleic acid is immobilized on nylon membranes, the blocking agents are often omitted from the hybridization solution, since high

TABLE 9.1 Blocking Agents Used to Suppress Background in Hybridization Experiments

Agent	Recommended uses
Denhardt's reagent	northern hybridizations hybridizations using RNA probes single-copy Southern hybridizations hybridizations involving DNA immobilized on nylon membranes

Denhardt's reagent (Denhardt 1966) is usually made up as a 50× stock solution, which is filtered and stored at −20°C. The stock solution is diluted tenfold into prehybridization buffer (usually 6× SSC or 6× SSPE containing 0.5% SDS and 100 μg/ml denatured, fragmented salmon sperm DNA). 50× Denhardt's reagent contains 5 g of Ficoll (Type 400, Pharmacia), 5 g of polyvinylpyrrolidone, 5 g of bovine serum albumin (Fraction V; Sigma), and H_2O to 500 ml.

BLOTTO	Grunstein/Hogness hybridization Benton/Davis hybridization all Southern hybridizations other than single-copy dot blots

1× BLOTTO (Bovine Lacto Transfer Technique Optimizer; Johnson et al. 1984) is 5% nonfat dried milk dissolved in water containing 0.02% sodium azide. It should be stored at 4°C and diluted 25-fold into prehybridization buffer before use. BLOTTO should not be used in combination with high concentrations of SDS, which will cause the milk proteins to precipitate. If background hybridization is a problem, NP-40 may be added to the hybridization solution to a final concentration of 1%. BLOTTO should not be used as a blocking agent in northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase.

Caution: Sodium azide is poisonous. It should be handled with great care, wearing gloves, and solutions containing it should be clearly marked.

Heparin	Southern hybridization in situ hybridization

Heparin (Sigma H-7005 porcine grade II or equivalent) is dissolved at a concentration of 50 mg/ml in 4× SSPE or 4× SSC and stored at 4°C. It is used as a blocking agent at a concentration of 500 μg/ml in hybridization solutions containing dextran sulfate; in hybridization solutions that do not contain dextran sulfate, heparin is used at a concentration of 50 μg/ml (Singh and Jones, 1984).

Denatured, fragmented salmon sperm DNA	Southern and northern hybridizations

Salmon sperm DNA (Sigma type III sodium salt) is dissolved in water at a concentration of 10 mg/ml. If necessary, the solution is stirred on a magnetic stirrer for 2–4 hours at room temperature to help the DNA to dissolve. The concentration of NaCl is adjusted to 0.1 M, and the solution is extracted once with phenol and once with phenol:chloroform. The aqueous phase is recovered, and the DNA is sheared by passing it 12 times rapidly through a 17-gauge hypodermic needle. The DNA is precipitated by adding 2 volumes of ice-cold ethanol. It is then recovered by centrifugation and redissolved at a concentration of 10 mg/ml in water. The OD_{260} of the solution is determined and the exact concentration of the DNA is calculated. The solution is then boiled for 10 minutes and stored at −20°C in small aliquots. Just before use, the solution is heated for 5 minutes in a boiling-water bath and then chilled quickly in ice water. Denatured, fragmented salmon sperm DNA should be used at a concentration of 100 μg/ml in prehybridization solutions.

concentrations of protein are believed to interfere with the annealing of the probe to its target. This quenching of the hybridization signal is particularly noticeable when oligonucleotides or probes less than 100 nucleotides in length are used.

7. In the presence of 10% dextran sulfate or 10% polyethylene glycol, the rate of hybridization is accelerated approximately tenfold (Wahl et al. 1979; Renz and Kurz 1984; Amasino 1986) because nucleic acids are excluded from the volume of the solution occupied by the polymer and their effective concentration is therefore increased. Although dextran sulfate and polyethylene glycol are useful in circumstances where the rate of hybridization is the limiting factor in detecting rare sequences (e.g., northern or genomic Southern blots), they are of no benefit when screening bacterial colonies or bacterial plaques. In addition, they can sometimes lead to high backgrounds, and hybridization solutions containing them are always difficult to handle because of their viscosity. We therefore recommend that dextran sulfate and polyethylene glycol not be used unless the rate of hybridization is very slow, the filter contains very small amounts of DNA, or the amount of radiolabeled probe is limiting.

8. To maximize the rate of annealing of the probe with its target, hybridizations are usually carried out in solutions of high ionic strength ($6 \times$ SSC or $6 \times$ SSPE) at a temperature that is 20–25°C below the melting temperature (T_m). Both solutions work equally well when hybridization is carried out in aqueous solvents. However, when formamide is included in the hybridization buffer, $6 \times$ SSPE is preferred because of its greater buffering power.

9. In general, the washing conditions should be as stringent as possible (i.e., a combination of temperature and salt concentration should be chosen that is approximately 12–20°C below the calculated T_m of the hybrid under study). The temperature and salt conditions can often be determined empirically in preliminary experiments in which samples of genomic DNA immobilized on filters are hybridized to the probe of interest and then washed under conditions of different stringencies.

10. To minimize background problems, it is best to hybridize for the shortest possible time using the minimum amount of probe. For Southern hybridization of mammalian genomic DNA where each lane of the gel contains 10 μg of DNA, 10–20 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater) should be used and hybridization should be carried out for 12–16 hours at 68°C in aqueous solution or for 24 hours at 42°C in 50% formamide. For Southern hybridization of fragments of cloned DNA where each band of the restriction digest contains 10 ng of DNA or more, much less probe is required. Typically, hybridization is carried out for 6–8 hours using 1–2 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater).

11. *Useful facts:*

   a. The T_m of the hybrid formed between the probe and its target may be estimated from the following equation (Bolton and McCarthy 1962):

$$T_\mathrm{m} = 81.5°C + 16.6(\log_{10}[\mathrm{Na}^+]) + 0.41(\text{fraction G} + \text{C}) - 0.63(\% \text{ formamide}) - (600/l)$$

where l = the length of the hybrid in base pairs.
This equation is valid for:

- Concentrations of Na^+ in the range of 0.01 M to 0.4 M. It predicts T_m less accurately in solutions of higher $[\mathrm{Na}^+]$.

- DNAs whose G + C content is in the range of 30% to 75%. Note that the depression of T_m in solutions containing formamide is greater for poly(dA:dT) (0.75°C/1% formamide) and less for DNAs rich in poly(dG:dC) (0.50°C/1% formamide) (Casey and Davidson 1977).

The equation applies to the "reversible" T_m that is defined by optical measurement of hyperchromicity at OD_{257}. The "irreversible" T_m, which is more important for autoradiographic detection of DNA hybrids, is usually 7–10°C higher than that predicted by the equation.
Similar equations have been derived for:

i. RNA probes hybridizing to immobilized RNA (Bodkin and Knudson 1985)

$$T_\mathrm{m} = 79.8°C + 18.5(\log_{10}[\mathrm{Na}^+]) + 0.58(\text{fraction G} + \text{C}) + 11.8(\text{fraction G} + \text{C})^2 - 0.35(\% \text{ formamide}) - (820/l)$$

ii. DNA:RNA hybrids (Casey and Davidson 1977)

$$T_\mathrm{m} = 79.8°C + 18.5(\log_{10}[\mathrm{Na}^+]) + 0.58(\text{fraction G} + \text{C}) + 11.8(\text{fraction G} + \text{C})^2 - 0.50(\% \text{ formamide}) - (820/l)$$

Comparison of these equations shows that the relative stability of nucleic acid hybrids decreases in the following order: RNA:RNA (most stable), RNA:DNA (less stable), and DNA:DNA (least stable). In aqueous solutions, the T_m of a DNA:DNA hybrid is approximately 10°C lower than that of the equivalent RNA:RNA hybrid. In 80% formamide, the T_m of an RNA:DNA hybrid is approximately 10°C higher than that of the equivalent DNA:DNA hybrid.

b. The T_m of a double-stranded DNA decreases by 1–1.5°C with every 1% decrease in homology (Bonner et al. 1973).

The above equations apply only to hybrids greater than 100 nucleotides in length. The behavior of oligonucleotide probes is described in detail in Chapter 11.

For a general discussion of hybridization of nucleic acids bound to solid supports, see Meinkoth and Wahl (1984).

Hybridization of Radiolabeled Probes to Nucleic Acids Immobilized on Nitrocellulose Filters or Nylon Membranes

Although the method given below deals with RNA or DNA immobilized on nitrocellulose filters, only slight modifications are required to adapt the procedure to nylon membranes. These modifications are noted at the appropriate places in the text.

1. Prepare the prehybridization solution appropriate for the task at hand. Approximately 0.2 ml of prehybridization solution will be required for each square centimeter of nitrocellulose filter or nylon membrane.

   The prehybridization solution should be filtered through a 0.45-micron disposable cellulose acetate filter (Schleicher and Schuell Uniflow syringe filter No. 57240 or equivalent).

Prehybridization solutions

For detection of low-abundance sequences:

Either
6× SSC (or 6× SSPE)
5× Denhardt's reagent
0.5% SDS
100 µg/ml denatured, fragmented salmon sperm DNA

or
6× SSC (or 6× SSPE)
5× Denhardt's reagent
0.5% SDS
100 µg/ml denatured, fragmented salmon sperm DNA
50% formamide

For preparation of Denhardt's reagent and denatured, fragmented salmon sperm DNA, see Table 9.1.

Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by stirring on a magnetic stirrer with Dowex XG8 mixed-bed resin for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

For detection of moderate- or high-abundance sequences:

Either
6× SSC (or 6× SSPE)
0.05× BLOTTO

or
6× SSC (or 6× SSPE)
0.05× BLOTTO
50% formamide

For preparation of BLOTTO, see Table 9.1.

When ^{32}P-labeled cDNA or RNA is used as a probe, poly(A)$^{+}$ RNA at a concentration of 1 μg/ml may be included in the prehybridization and hybridization solutions to prevent the probe from binding to T-rich sequences that are found fairly commonly in eukaryotic DNA.

2. Float the nitrocellulose filter or nylon membrane containing the target DNA on the surface of a tray of 6 × SSC (or 6 × SSPE) until it becomes thoroughly wetted from beneath. Submerge the filter for 2 minutes.

3. Slip the wet filter into a heat-sealable bag (e.g., Sears Seal-A-Meal or equivalent). Add 0.2 ml of prehybridization solution for each square centimeter of nitrocellulose filter or nylon membrane.

   Squeeze as much air as possible from the bag. Seal the open end of the bag with the heat sealer. Incubate the bag for 1–2 hours submerged at the appropriate temperature (68°C for aqueous solvents; 42°C for solvents containing 50% formamide).

   Often, small bubbles of air form on the surface of the filter as the temperature of the prehybridization solution increases. It is important that these bubbles be removed by occasionally agitating the fluid in the bag; otherwise, the components of the prehybridization solution will not be able to coat the filter evenly. This problem can be minimized by heating the prehybridization solution to the appropriate temperature before adding it to the bag.

4. If the radiolabeled probe is double-stranded, denature it by heating for 5 minutes at 100°C. Single-stranded probe need not be denatured. Chill the probe rapidly in ice water.

   Alternatively, the probe may be denatured by adding 0.1 volume of 3 N NaOH. After 5 minutes at room temperature, transfer the probe to ice water and add 0.05 volume of 1 M Tris · Cl (pH 7.2) and 0.1 volume of 3 N HCl. Store the probe in ice water until it is needed.

   For Southern hybridization of mammalian genomic DNA where each lane of the gel contains 10 μg of DNA, 10–20 ng/ml radiolabeled probe (sp. act. = 10^{9} cpm/μg or greater) should be used. For Southern hybridization of fragments of cloned DNA where each band of the restriction digest contains 10 ng of DNA or more, much less probe is required. Typically, hybridization is carried out for 6–8 hours using 1–2 ng/ml radiolabeled probe (sp. act. = 10^{9} cpm/μg or greater).

5. Working quickly, remove the bag containing the filter from the water bath. Open the bag by cutting off one corner with scissors. Add the denatured probe to the prehybridization solution, and then squeeze as much air as possible from the bag. Reseal the bag with the heat sealer so that as few bubbles as possible are trapped in the bag. To avoid radioactive contamination of the water bath, the resealed bag should be sealed inside a second, noncontaminated bag.

   When using nylon membranes, the prehybridization solution should be *completely* removed from the bag and immediately replaced with hybridization solution. The probe is then added and the bag is resealed.

Hybridization solution for nylon membranes

6× SSC (or 6× SSPE)
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA
50% formamide (if hybridization is to be carried out at 42°C)

6. Incubate the bag submerged in a water bath set at the appropriate temperature for the required period of hybridization.

7. Wearing gloves, remove the bag from the water bath and immediately cut off one corner. Pour out the hybridization solution into a container suitable for disposal, and then cut the bag along the length of three sides. Remove the filter and immediately submerge it in a tray containing several hundred milliliters of 2× SSC and 0.5% SDS at room temperature.

    Important: Do not allow the filter to dry out at any stage during the washing procedure.

8. After 5 minutes, transfer the filter to a fresh tray containing several hundred milliliters of 2× SSC and 0.1% SDS and incubate for 15 minutes at room temperature with occasional gentle agitation.

    If short oligonucleotides are used as probes, washing should be carried out only for brief periods (1–2 minutes) at the appropriate temperature. For a discussion of the stability of hybrids involving oligonucleotides, see Chapter 11.

9. Transfer the filter to a flat-bottom plastic box containing several hundred milliliters of fresh 0.1× SSC and 0.5% SDS. Incubate the filter for 30 minutes to 1 hour at 37°C with gentle agitation.

10. Replace the solution with fresh 0.1× SSC and 0.5% SDS, and transfer the box to a water bath set at 68°C for an equal period of time. Monitor the amount of radioactivity on the filter using a hand-held minimonitor. The parts of the filter that do not contain DNA should not emit a detectable signal. You should not expect to pick up a signal on the minimonitor from filters containing mammalian DNA that has been hybridized to single-copy probes.

11. Briefly wash the filter with 0.1× SSC at room temperature. Remove most of the liquid from the filter by placing it on a pad of paper towels.

12. Place the damp filter on a sheet of Saran Wrap. Apply adhesive dot labels marked with radioactive ink to several asymmetric locations on the Saran Wrap. These markers serve to align the autoradiograph with the filter. Cover the labels with Scotch Tape. This prevents contamination of the film holder or intensifying screen with the radioactive ink.

    Radioactive ink is made by mixing a small amount of ^{32}P with waterproof black drawing ink. We find it convenient to make the ink in three grades: very hot

(>2000 cps on a hand-held minimonitor), hot (>500 cps on a hand-held minimonitor), and cool (>50 cps on a hand-held minimonitor). Use a fiber-tip pen to apply ink of the desired hotness to the adhesive labels. Attach radioactive-warning tape to the pen, and store it in an appropriate place.

13. Cover the filter with a second sheet of Saran Wrap, and expose the filter to X-ray film (Kodak XAR-2 or equivalent) to obtain an autoradiographic image (see Appendix E). The exposure time should be determined empirically. However, single-copy sequences in mammalian genomic DNA can usually be detected after 16–24 hours of exposure at −70°C with an intensifying screen.

Hybridization of Radiolabeled Oligonucleotides to Genomic DNA

Oligonucleotide probes as short as 17 nucleotides in length may be used to detect single-copy sequences in restriction digests of eukaryotic genomic DNA that have been transferred to solid supports. As discussed in Chapter 11, hybrids of this length are stable enough to be detected in practice only if they are perfectly matched. Duplexes with a single base-pair mismatch are significantly less stable and dissociate at a lower temperature than their perfectly matched counterparts (Wallace et al. 1979; Ikuta et al. 1987). It has therefore been possible to use oligonucleotides of defined sequence to probe fetal DNA for the presence of specific point mutations that cause conditions such as sickle-cell anemia (Conner et al. 1983), certain thalassemias (Orkin et al. 1983; Pirastu et al. 1983), and α_1-antitrypsin deficiency (Kidd et al. 1983); to screen DNA extracted from tumor cells for mutations in oncogenes (Bos et al. 1984, 1985, 1987; Forrester et al. 1987; Rodenhuis et al. 1987); and to analyze highly polymorphic loci, for example, the major histocompatibility complex class I genes (Geliebter et al. 1986).

The methods used when hybridizing with oligonucleotide probes are similar to those described earlier in this chapter. However, attention should be paid to the following points:

1. Because of the small size of the target sequence, a minimum of 30 μg of mammalian genomic DNA should be applied to each lane of the agarose gel.

2. The sequences of oligonucleotides used as probes should be long enough to be unique within the target genome (17 nucleotides for the mammalian genome) and short enough to allow the detection of mismatches under the conditions of hybridization used. Typically, oligonucleotides used for screening mammalian genomic DNA are 19–21 nucleotides in length.

3. When used to detect point mutations, oligonucleotides are used in pairs; one member of the pair is perfectly homologous to the mutated gene sequence and the other is homologous to the wild-type sequence. Usually, the members of the pair differ in sequence by only one nucleotide. Before embarking on an analysis of genomic DNA with these probes, it is essential to establish hybridization and washing conditions using cloned fragments of DNA of known sequence that are homologous to each member of the pair of oligonucleotides. These methods are discussed in detail in Chapter 11. Reconstruction experiments, in which known amounts of the control DNAs are added to a large excess of genomic DNA (at least 30 μg), are then used to test the sensitivity of the system.

4. Oligonucleotides are radiolabeled by [γ-^{32}P]ATP and bacteriophage T4 polynucleotide kinase (see Chapter 11). These probes tend to hybridize nonspecifically to high-molecular-weight DNA immobilized on nitrocellulose filters or nylon membranes, producing a smear toward the top of the autoradiograph. It is therefore important to choose a restriction enzyme (or a combination of restriction enzymes) that yields a hybridizing fragment whose size is not greater than 5 kb.

5. After electrophoresis, the fragments of genomic DNA may be transferred to a solid support by the conventional Southern transfer technique or immobilized within the agarose gel itself by dehydration (Studencki and Wallace 1984). Although DNA immobilized within the gel appears to give somewhat stronger hybridization signals than DNA attached to a solid support, it cannot be hybridized sequentially to many different probes. This is a severe disadvantage when the amount of genomic DNA is limited (as is often the case in prenatal diagnosis, for example). We therefore recommend that the genomic DNA be transferred to a nylon membrane such as Nytran (Schleicher and Schuell) or GeneScreen (du Pont).

6. Wherever possible, negative and positive hybridization controls should be included in each gel.

7. Oligonucleotides may also be used to detect rare transcripts in northern blots that contain 30 μg of total cellular RNA (Zeff et al. 1986) or 5 μg of poly(A)$^+$ RNA (Geliebter et al. 1986).